ISOLATION AND EXPRESSION OF HEMOLYSIN E (HlyE) FROM *Salmonella enterica* serovar Typhi (*S.* Typhi) ISOLATES

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

AP	Alkaline persulfate
bp	Base pair
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IMAC	Immobilized metal affinity chromatography
IPTG	Isopropyl-beta-D-thiogalactopyranoside
kb	Kilobase
kDa	KiloDalton
L	Liter
mA	Milliampere
mg	Milligram
mL	Milliliter
MW	Molecular Weight
nm	Nanometer
OD	Optical Density
PCR	Polymerase Chain Reaction
pH	Potential Hydrogeni
RE	Restriction enzyme
rpm	Revolutions per minute
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SPI	Salmonella Pathogenecity Island
Taq DNA polymerase	*Thermus aquaticus* DNA polymerase
TEMED	N, N, N', N'- tetramethylethylenediamine
UV	Ultraviolet
V	Voltage
µL	Microliter
µg	Microgram

PENGASINGAN DAN PENGEKSPRESAN HEMOLISIN E (HlyE) DARIPADA *Salmonella enterica* serovar Typhi (*S.* Typhi) ISOLAT

ABSTRAK

Analisa perbandingan proteomik *Salmonella enterica* serovar Typhi (*S.* Typhi) dan *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) menunjukkan sekumpulan protein yang diekspres unik terhadap *S.* Typhi. Salah satu daripada protein tersebut adalah hemolisin E (HlyE). Gen *hlyE* ini adalah spesifik untuk perumah manusia, tiada atau berubah fungsi dalam *S.* Typhimurium. Gen *hlyE* merupakan gen yang terdapat pada 'Salmonella Pathogenecity Island (SPI) 18'. Dalam kajian ini, kehadiran dan fungsi gen *hlyE* di dalam isolat klinikal *S.* Typhi telah ditentukan. Analisis PCR dan jujukan DNA menunjukkan bahawa *S.* Typhi mempunyai keseluruhan salinan jujukan bagi gen *hlyE*. Walaupun gen *hlyE* hadir di dalam isolat *S.* Typhi, bakteria ini tidak menunjukkan zon hemolitik pada agar darah kuda. Peningkatan aktiviti hemolitik telah dilihat apabila *S.* Typhi dikultur pada agar darah kuda menggunakan asai cakera-difusi dengan kehadiran ampisilin. Protein periplasmik yang diekstrak daripada *S.* Typhimurium dan *S.* Typhi (strain vaksin dan isolat klinikal) menunjukkan kehadiran protein 34 kDa iaitu merupakan anggaran saiz untuk HlyE. Ekstrak protein periplasmik daripada *S.* Typhi menunjukkan aktiviti hemolitik yang kuat pada agar darah. Protein rekombinan HlyE menunjukkan reaksi positif terhadap IgG sera pesakit demam kepialu, ini menunjukkan penghasilan HlyE yang reaktif ketika infeksi. Protein rekombinan HlyE yang ditulenkan ini mempunyai kesan sitotoksik terhadap sel U937 manusia. Kajian ini memberikan informasi asas

terhadap aktiviti gen *hlyE* dan pengetahuan tambahan dalam kajian kepatogenan *S.* Typhi terhadap manusia

ISOLATION AND EXPRESSION OF HEMOLYSIN E (HlyE)

FROM *Salmonella enterica* serovar Typhi (*S.* Typhi) ISOLATES

ABSTRACT

Comparative proteomic analysis of *Salmonella enterica* serovar Typhi (*S.* Typhi) and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) revealed a subset of highly expressed proteins unique to *S.* Typhi. One of these proteins is hemolysin E (HlyE). The *hlyE* gene is necessary for human-host specificity, which is absent or functionally altered in *S.* Typhimurium. *hlyE* gene belongs to *Salmonella* Pathogenecity Island (SPI) 18. In this study, the presence and the function of *hlyE* gene in *S.* Typhi clinical isolates was determined. PCR and DNA sequence analyses showed that *S.* Typhi, serovar that is specific for human, harbors an intact copy of *hlyE* gene. Despite the presence of *hlyE* gene in *S.* Typhi isolates, the bacteria did not display haemolytic activity when cultured on horse blood agar plates. Increased haemolytic activity was observed when *S.* Typhi was grown on horse agar plates in disk-diffusion assay in the presence of ampicillin disk. Crude periplasmic proteins extracted from *S.* Typhimurium and *S.*Typhi (vaccine strain and clinical isolates) showed the presence of 34 kDa proteins, which is an expected size for HlyE. The crude protein extracts from *S.* Typhi showed strong haemolytic activity on the blood agar plates. HlyE recombinant protein showed positive reaction against IgG of sera from typhoid patients, suggesting reactive HlyE production during infection. The purified HlyE recombinant proteins have a cytotoxic activity towards U937 human monocytic cell line. This research provides fundamental information on the activity

of *hlyE* gene product and contributes additional knowledge in the study of *S.* Typhi pathogenesis in human.

CHAPTER 1

INTRODUCTION

1.1 *Salmonella* and disease: An overview

'Salmonella' was named after Daniel Elmer Salmon, an American veterinary pathologist, who discovered *Salmonella* bacterium isolated from pigs in 1885. Currently, there are 2579 classified serovars of *Salmonella enterica* (Grimont & Patrick, 2007) which are differentiated by their antigenic characteristics and host range (Grimont & Patrick, 2007; Popoff & Minor, 1997). The nomenclature system used for the genus *Salmonella* is based on recommendations from the Centers for Disease Control and Prevention (CDC), USA (Brenner *et al.*, 2000). The complete names of these serovars are commonly abbreviated such as '*Salmonella enterica* serovar Typhi' is commonly referred as *Salmonella* Typhi or *S.* Typhi (Judicial, 2005). The various *Salmonella enterica* serovars infect a wide range of vertebrate animals. Several serovars have very narrow and restricted host. For example, humans are host-specific for serovar Typhi whereas serovar Pullorum exclusively infects chicken. These host-specific serovars cause a systemic illness that could be fatal if left untreated. In contrast to *S.* Typhi, *S.* Typhimurium, infects a wide range of hosts including humans and other vertebrates.

Infections of *S. enterica* in human may display different clinical manifestation depending on the *Salmonella enterica* serovars. There are two main clinical manifestation associated with *Salmonella* infections in humans. First, *S.* Typhi and *S.*

Paratyphi cause systemic infection called typhoid fever. This enteric fever is a life-threatening disease and display several symptoms like prolonged fever (38°C and above) followed by malaise, anorexia and nausea. 1–3% of hospitalized patients faced the more serious complication, which is intestinal perforation. After traversing the intestinal mucosa, the causative bacteria then disseminate, resulting in secondary infections of the liver, spleen, bone narrow, gallbladder and Peyer's patches of the terminal ileum (Parry *et al*., 2002). The second is gastrointestinal disease, the more common clinical outcome of *Salmonella* infection in humans caused by non-typhoidal *Salmonella*. This non-typhoidal salmonellosis is commonly caused by *S*. Typhimurium and *S*. Enteritidis, which results in self-limiting disease such as nausea, vomiting, abdominal cramping and diarrhea. The non-typhoidal *Salmonella* are primarily transmitted to human directly or indirectly from animal sources and mostly via foodborne. In some cases the sources of this infection are pets, direct personal contact, nosocomial infection, waterborne and contaminated drugs and solutions (Hohman, 2001; Ohl & Miller, 2001). Patients less than 3 months old, greater than 50 years old, and patients with diabetes, malignancy, rheumatologic disorders, HIV infection and immunosuppression, are at risk of getting gastrointestinal salmonellosis. About 5% of non-typhoidal infections are associated with bacteremia (Hohman, 2001; Pang *et al*., 1995).

1.2 Typhoid fever

Typhoid fever is a global health problem. According to World Health Organization (WHO), approximately 216 000 to 600 000 (1-4%) deaths are reported yearly from 21 million registered typhoid cases worldwide. It is estimated that 90% of the death cases occur in Asia. Ministry of Health (MOH) Malaysia reported that the annual

incidences of typhoid in Malaysia for the past 10 years (1998–2007) is below 5 cases per 100 000 population, which is classified as low endemic region for typhoid fever. Kelantan has the highest annual incidence when compared to other states in Malaysia. From April to June 2005, an outbreak occurred in Kelantan with 735 reported cases and 2 deaths (MOH, 2007).

A person is confirmed to have typhoid fever when he has a fever (38°C and above) for at least 3 days with a laboratory confirmed positive culture (blood, bone marrow or stool) for *S.* Typhi (WHO, 2003). Mode of infection is by ingestion of food or water contaminated with fecal that contains *S.* Typhi. The infection is transmitted from person to person through poor hygiene practices and sewage contamination of water supply (MOH, 2007). Acute systemic illness is characterized by prolonged fever, abdominal pain, and persistent bacteremia. About 10 to 20% of patients showed acute diarrhea symptom after ingestion of *S.* Typhi. This may last for several days. On the second week of infection, the fever continues and the patient may appear severely ill. Gastrointestinal complications, such as bleeding or perforation may occur at any time but usually during the third week of infection (Levine, 2009). Common physical symptoms for typhoid patients are prolonged fever, brown coated tongue, confusion, decreased auditory acuity and nuchal rigidity (Crum, 2003). Rose spots on abdomen and chest may develop on some patients (Levine, 2009).

1.3 *Salmonella enterica* serovar Typhi

S. Typhi is a rod-shape, Gram negative, facultative anaerobic, non-encapsulated, flagellated bacilli belonging to the family of *Enterobacteriaceae*. It is motile with peritrichous flagella. *S.* Typhi is identified in the laboratory by culturing on selective

media followed by several biochemical and serological tests. Suspected colony obtained from selective media is further distinguished from other *Salmonella* serotypes by its biochemical properties and agglutination with specific antisera. For example, on Salmonella Shigella (SS) agar, Salmonellae produce lactose non-fermenting colonies with black centres. The colony with such appearance was chosen for biochemical tests screening as described in Table 1.1. In serological test, *S.* Typhi is positive for LPS antigen O9 and O12, protein flagella antigen H-d and polysaccharide capsular antigen Vi (WHO, 2003).

Table 1.1: Biochemical tests for differentiation of *S.* Typhi from related subspecies (adapted from WHO, 2003)

Organism	Kliger's Iron Agar Test				Motility, Indole, Urea Tests			Citrate Test
	Slant	Butt	H_2S	Gas	Motility	Indole	Urea	
S. Typhi	Alkaline	Acid	Wk+	-	+	-	-	-
S. Paratyphi A	Alkaline	Acid	-	+	+	-	-	-
Other *Salmonella* sp.	Alkaline	Acid	V	V	+	-	-	V

'+' = Positive

'-' = Negative

Wk + = Weak positive

V = Variable result

H_2S = Hydrogen sulphide

1.4 Laboratory diagnosis of typhoid fever

1.4.1 Identification of *S*. Typhi by culture method

The definitive diagnosis of typhoid fever depends on the isolation of *S*. Typhi from blood, bone marrow or a specific anatomical lesion from typhoid patients. Bone marrow aspirate culture is the gold standard for the diagnosis of typhoid fever and is principally important for patients who have been previously treated, have a long history of illness and have been getting negative for blood culture at the recommended volume of blood (WHO, 2003). However, bone marrow aspirate samples are difficult to obtain and relatively (Bhutta, 2006). Thus, blood culture is the best option to diagnose typhoid fever, as more than 80% of typhoid patients have the causative bacteria in their blood (WHO, 2003).

1.4.2 Serological diagnostic methods

Serological diagnostic tests give a rapid diagnosis of typhoid fever compared to bacterial culture method, which requires 3 to 5 days to perform. There are several serological methods widely being used to diagnose typhoid fever. Felix-Widal test works by detecting agglutinating antibodies against O and H antigens. However, the test only gives moderate sensitivity and specificity. It produces false negative results for up to 30% of culture-positive cases of typhoid fever (WHO, 2003). Moreover, other *Salmonella* serotypes also posses the same O and H antigens similar to *S*. Typhi (WHO, 2003; Grimont & Patrick, 2007) and there are cross-reacting epitopes between *S*. Typhi with other *Enterobacteriaceae* (WHO, 2003). These factors can lead to false positive results. However, the test is still being used in many areas since it is an affordable diagnostic method (Baba *et al*., 2013; Ley *et al*., 2010).

TYPHIDOT® test kit is developed under USM license based on the principle of dot-blot enzyme immune-assay (Dot-EIA), for detecting IgM and IgG antibodies specific for a 50 kDa antigen of *S.* Typhi (Ismail *et al*., 1991a). This protein is an outer membrane protein and is found to be a specific protein to *S.* Typhi (Ismail *et al*., 1991b). Dot EIA test offers simplicity, speedity/rapidity, specificity (75%), sensitivity (95%) and cost-effective (Choo *et al*., 1994). However, in convalescence or reinfection cases, the level of IgG is boosted by the secondary immune response, which mask the IgM. In order to increase the diagnostic accuracy, TYPHIDOT-M was later developed where the original TYPHIDOT® test was modified by inactivating total IgG in the sera sample. Inactivation of IgG removes competitive binding and allows access of the antigen to bind to the specific IgM (Bhutta & Mansurali, 1999). This test was reported to be useful in typhoid endemic areas, since it can be differentiate between convalescence and acute cases. This test was reported to have 92% sensitivity and 100% specificity in independent evaluation studies for all age groups of typhoid fever (Bhutta, 2006).

1.5 Pathogenicity of *S*. Typhi

Once the *S.* Typhi enters the host via ingestion of contaminated food or water, it passes through the stomach and the gastric acid barrier, then it reaches a site where it can invade the small intestine epithelial cells. *S.* Typhi invades the epithelium through two ways, which are by invasion of microfold (M) cells of Peyer's patches and by direct translocation across the absorptive epithelial cells. After penetration into the intestinal mucosa, the bacteria are immediately engulfed by macrophages but resist from being killed. Through these vehicles, they are able to enter mesenteric lymph nodes. The bacteria multiply until the critical microbial load is sufficient to